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ABSTRACT

We hypothesized that tobacco smoke induces alterations to the 3-OH fatty acids present in lipid A in a manner consistent with a microflora of reduced inflammatory potential. Whole saliva samples and full-mouth clinical periodontal recordings were obtained from persons with (22 smokers; 15 non-smokers) and without (14 smokers; 15 non-smokers) chronic periodontitis. Clear differences in the contributions of multiple saturated 3-OH fatty acid species were noted in the group with disease compared with healthy individuals. Increases in the long-chain fatty acids associated with anaerobic bacterial periodontopathogens, particularly 3-OH-C_{17:0} (146.7%, relative to controls), were apparent. Significant reductions in the 3-OH fatty acids associated with the consensus (high potency) enteric LPS structure (3-OH-C_{12:0} and 3-OH-C_{14:0}; 33.3% and 15.8% reduction, respectively) were noted in smokers compared with non-smokers with chronic periodontitis. Thus, smoking is associated with specific structural alterations to the lipid-A-derived 3-OH fatty acid profile in saliva that are consistent with an oral microflora of reduced inflammatory potential. These findings provide much-needed mechanistic insight into the established clinical conundrum of increased infection with periodontal pathogens but reduced clinical inflammation in smokers.

KEY WORDS: inflammation, lipid A, lipopolysaccharide, periodontitis, saliva, smoking.

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Fatty Acid Profiles in Smokers with Chronic Periodontitis

INTRODUCTION

Compared with non-smokers, smokers exhibit increased prevalence of periodontal disease (Calsina *et al.*, 2002), increased severity of periodontal disease (Mullally, 2004; Baljoon, 2005), earlier onset of disease (Haber, 1994; Mullally, 2004), increased rates of disease progression (Bergström, 2004; Fisher *et al.*, 2007), and less favorable response to treatment (Ah *et al.*, 1994; Haffajee *et al.*, 1997); while former smokers are at a lower disease risk than current smokers (Bostrom *et al.*, 2001). The negative effects of smoking on periodontal health also appear to be dose-related (Gonzalez *et al.*, 1996; Calsina *et al.*, 2002). Despite such increased disease susceptibility, overt gingival inflammation (edema, redness, exudation of crevicular fluid, bleeding on probing, etc.) and several biochemical inflammatory biomarkers (most notably the pro-inflammatory cytokines) have long been known to be suppressed in smokers compared with non-smokers (Pindborg, 1947; McMurray *et al.*, 1969; Scott and Singer, 2004; Palmer *et al.*, 2005; Johnson and Guthmiller, 2007). This phenomenon represents the classic “clinical conundrum” in periodontics.

Several reviews highlight that the mechanisms predisposing tobacco smokers to periodontitis have yet to be defined (Barbour *et al.*, 1997; Kinane and Chestnutt, 2000; Scott *et al.*, 2001; Johnson and Hill, 2004; Mullally, 2004; Palmer *et al.*, 2005). Attention has been primarily directed toward tobacco-host response interactions (Nair *et al.*, 2003; Palmer *et al.*, 2005), with minimal studies examining tobacco-bacterial interactions.

Periodontal inflammation is induced by the interactions of microbial-associated molecular patterns (MAMPs) with pattern recognition receptors (PRRs) expressed by innate cells. LPS is a key MAMP that is contained in the outer leaflet of the outer membrane of Gram-negative bacteria, that interacts with TLR-2 and/or TLR-4 (Sing *et al.*, 2002; Ogawa *et al.*, 2007; Berezow *et al.*, 2009), and is a prominent mediator of periodontal inflammation. The lipid A portion of the LPS molecule is relatively conserved and is the moiety that engenders inflammatory potential to LPS (Berezow *et al.*, 2009). Lipid A potency is highly variable and is determined by the phosphorylation state and, in particular, by the number and length of the 3-OH fatty acid chains (Ogawa *et al.*, 1996; Schromm *et al.*, 2000; Rebeil *et al.*, 2004). *Escherichia coli* LPS (biphosphorylated, hexa-acylated, C12-C14), the classic TLR4 agonist, represents the most potent pro-inflammatory structural form identified to date (Berezow *et al.*, 2009).

Structural alterations in LPS, a feature of several important human pathogens, are often triggered by variant environmental stimuli (Rebeil *et al.*, 2004; Al-Qutub *et al.*, 2006). Tobacco smoke, the major environmental risk factor for periodontitis, obviously represents a potent environmental stressor.

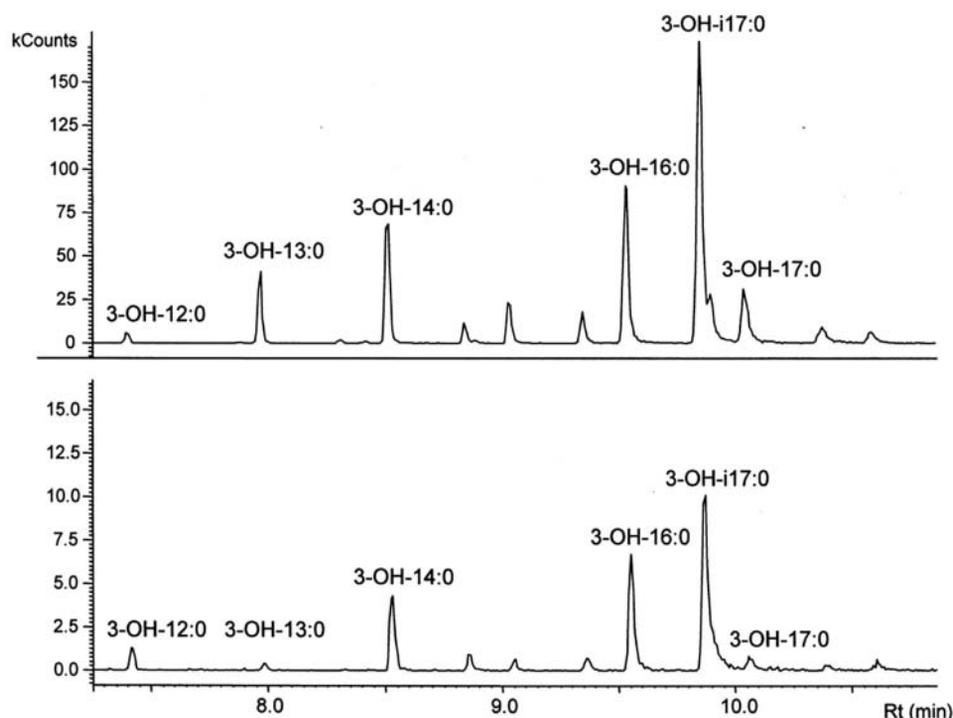


Figure 1. 3-OH fatty acid profiles of saliva from a current non-smoker with chronic periodontitis (lower) and a smoker with chronic periodontitis (upper).

Tobacco smoke has been shown to cause shifts in the microbial species that comprise dental plaque (Zambon *et al.*, 1996; Umeda *et al.*, 1998; Kamma *et al.*, 1999; Khaira *et al.*, 2000; Shiloah *et al.*, 2000; Haffajee and Socransky, 2001; van Winkelhoff *et al.*, 2001; Gomes *et al.*, 2008). We hypothesized, therefore, that *in vivo* tobacco smoke exposure could lead to an overall reduction in the inflammatory potential of periodontal bacteria by altering the ratios of lipid A subtypes. This could occur by tobacco-induced alterations in either the presence or numbers of different Gram-negative bacterial species in the oral cavity, by inducing structural shifts in the type of lipid A produced by the extant microflora, or both.

Therefore, we examined the ratios of 3-OH fatty acids in saliva samples from smoking and non-smoking individuals with chronic periodontitis.

MATERIALS & METHODS

Materials

Cotinine enzyme immunoassays were purchased from Cozart (Abingdon, UK). Methanol, toluene, and sulphuric acid came from Merck (Darmstadt, Germany). Heptane, pyridine, and N,O-bis (trimethylsilyl) trifluoroacetamide were bought from Sigma-Aldrich (Madrid, Spain).

Study Population

Thirty-seven otherwise healthy individuals with chronic periodontitis (CP) (22 smokers, 15 non-smokers) and 29 systemically and periodontally healthy individuals (15 smokers, 14

non-smokers) were recruited from among patients presenting to the School of Dentistry, Ege University, between February 2007 and July 2009. The study protocol was approved by the local ethics committee, with written informed consent obtained from each individual. Medical and dental histories were obtained. Patients with medical disorders unrelated to periodontal disease, but likely to influence study outcomes, or with antibiotic or periodontal treatment in the previous 6 mos were excluded from the study.

CP patients were diagnosed in accordance with the following criteria (Armitage, 1999): a minimum of 4 teeth in each jaw with a probing depth (PD) of ≥ 5 mm; and clinical attachment level (CAL) of ≥ 4 mm; with $\geq 50\%$ alveolar bone loss in at least two quadrants. Periodontal health was defined as $\geq 90\%$ of the measured sites with PD < 3 mm and

CAL ≤ 1 mm; no bleeding on probing (BOP); and no radiographic signs of alveolar bone loss (*i.e.*, a distance of < 3 mm between the cement-enamel junction (CEJ) and bone crest at $> 95\%$ of the proximal tooth sites). Alveolar bone loss was assessed radiographically. The clinical and demographic characteristics of the study population are presented in Table 1.

Saliva Sampling

Unstimulated whole saliva samples (3-5 mL) were obtained by expectoration into polypropylene tubes prior to clinical measurements and stored at -40°C until analysis for cotinine and 3-OH fatty acid content.

Clinical Measurements

Dichotomous plaque index (\pm), PD, CAL (cement-enamel junction to the base of the probable pocket), and BOP (\pm after 15 sec) were assessed by precalibrated examiners (NB and BB) at 6 sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual locations) on each tooth present, except the third molars, using a Williams probe.

Assessment of Tobacco Smoke Exposure

Tobacco exposure was initially established by self-report and, subsequently, by measurement of salivary cotinine concentrations by a commercial enzyme immunoassay (cotinine EIA; Cozart, Abingdon, UK), according to the manufacturer's instructions. We used a cut-off of 10 ng/mL salivary cotinine to differentiate current smokers and current non-smokers biochemically.

Table 1. Demographic Variables and Clinical Periodontal Measurements in the Study Groups

	CPS (n = 22)	H-S (n = 14)	CP-NS (n = 15)	H-NS (n = 15)
Age (yrs, SD)	45.4 (6.0)	46.7 (4.3)	47.7 (7.8)	43.6 (5.6)
Gender (m/f)	16/6	4/10	9/6	4/11
Cigarettes/day	18.7, 10.4	16.1, 5.9	–	–
Yrs smoked	25.7, 7.4	21.6, 6.3	–	–
Cotinine	125.5, 124.8	92.3, 136.3	1.4, 0.8	1.5, 2.2
PD (mm)	4.2, 0.8	1.9, 0.4	3.6, 0.5	1.4, 0.6
CAL (mm)	5.5, 1.5	0.2, 0.4	4.2, 0.7	0.1, 0.2
PI	96.8, 14.9	2.9, 5.8	97.9, 4.3	6.5, 6.0
BOP	91.4, 17.1	2.0, 3.6	97.1, 6.6	5.7, 6.7
No. of teeth	20.1, 4.2	26.9, 2.1	22.2, 4.0	27.6, 1.0
Sites (%) > 5 mm	50.0, 25.3	0.0, 0.0	39.9, 17.9	0.0, 0.0

There were no statistical differences in the clinical parameters of smokers and non-smokers with periodontitis.

Establishment of Salivary 3-OH Fatty Acid Profiles

Saturated straight- and branched-chain 3-OH fatty acid profiles were determined in saliva, essentially as previously described (Larsson and Saraf, 1997; Sebastian and Larsson, 2003; Ferrando *et al.*, 2005). Briefly, pellets of centrifuged saliva samples (1500 x g, 15 min) were subjected to methanolysis by the dry methanol-hydrochloric-acid method (2-M, 80°C, 18 hrs) (Sebastian and Larsson, 2003; Ferrando *et al.*, 2005). Prior to analysis, 3-OH fatty acid methyl esters were transformed to trimethylsilyl (TMS) ethers by pyridine and N,O-bis (trimethylsilyl) trifluoroacetamide treatment (Sebastian and Larsson, 2003). Samples were kept at 8°C prior to gas chromatography–tandem mass spectroscopy (GC-MS-MS) analysis in a Saturn 2000 ion trap GC-MS (Varian, Walnut Creek, CA, USA) equipped with a fused-silica capillary column (CP-Sil 8 CB low bleed, 30 m x 0.25 mm ID, 0.25 µm film thickness). Samples, diluted in heptane, were injected in the splitless mode, and the temperature in the column rose from 90°C to 280°C at a rate of 20°C/min. The injector, transfer line, and ion trap were held at, respectively, 280°C, 290°C, and 180°C (Ferrando *et al.*, 2005). Ions of m/z 131 representing product ions of m/z 175 (resulting from cleavage of C3/C4 of the 3-OTMS methyl ester derivative) were monitored (Larsson and Saraf, 1997).

Statistical Analysis

Statistical significance between groups was evaluated by the *t* test or the Mann-Whitney test for normal and non-normal datasets, respectively, or by one-way non-parametric analysis of variance (ANOVA) and the Tukey multiple-comparison test, as appropriate, with the InStat program (Graph-Pad Software, San Diego, CA, USA). Significance was set at the level of $p < 0.05$.

RESULTS

Clinical Analyses

Demographic variables and mean values of clinical measurements are outlined in Table 1. Other than tobacco exposure, there were

Table 2. 3-Hydroxy Fatty Acid Composition [relative percentage; mean (\pm SD)] of Persons with Chronic Periodontitis and Healthy Control Individuals

3-Hydroxy Fatty Acid	Healthy	Periodontitis
3-OH-C _{12:0}	4.5 (3.1)	2.2 (1.3)***
3-OH-C _{13:0}	6.3 (4.0)	2.6 (1.7)***
3-OH-C _{14:0}	23.4 (7.3)	15.0 (4.0)***
3-OH-C _{15:0}	1.7 (0.7)	2.3 (1.0)**
3-OH-C _{15:0}	4.5 (2.4)	2.8 (1.3)***
3-OH-C _{16:0}	2.3 (0.7)	2.8 (0.9)*
3-OH-C _{16:0}	23.3 (3.8)	25.5 (8.7)
3-OH-C _{17:0}	29.4 (9.1)	43.1 (9.7)***
3-OH-C _{17:0}	4.5 (2.3)	3.8 (4.5)

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

no statistically significant differences in any variable measured between the smokers and non-smokers with periodontitis. As dictated by the inclusion criteria, all clinical variables were significantly worse in the groups with disease. The similarity in clinical periodontal measurements between the smoker and non-smoker chronic periodontitis patients was rather unexpected and perhaps due to rather low numbers of patients in the study groups and/or the high similarity in the demographic variables of the periodontitis groups. Nevertheless, such balance strengthens the clinical significance of altered lipid A profiles in smokers and non-smokers of similar disease levels.

Salivary 3-OH Fatty Acid Profiles in Persons with Chronic Periodontitis and Periodontally Healthy Individuals

The 3-hydroxy fatty acid composition [relative percentage; mean (\pm SD)] of individuals with CP and healthy control individuals is presented in Table 2. Dramatic shifts in the 3-OH were obvious between health and disease, which were statistically significant for 7 of the 9 fatty acids monitored (all but 3-OH C16

Table 3. 3-Hydroxy Fatty Acid Composition [relative percentage; mean (\pm SD)] of Smokers and Non-smokers with Healthy Periodontia and Chronic Periodontitis

3-OH Fatty Acid	Healthy Periodontia		Chronic Periodontitis	
	Non-smokers	Smokers	Non-smokers	Smokers
3-OH-C _{12,0}	4.0 (2.5)	5.4 (3.9)	2.7 (1.3)	1.8 (1.1)*
3-OH-C _{13,0}	4.6 (1.8)	8.6 (4.9)*	1.9 (1.0)	3.1 (1.9)*
3-OH-C _{14,0}	23.4 (7.0)	23.9 (8.2)	16.5 (3.6)	13.9 (3.9)#
3-OH-C _{15,0}	1.8 (0.7)	1.6 (0.6)	2.3 (1.0)	2.4 (0.9)
3-OH-C _{15,0}	3.9 (1.4)	5.2 (3.2)	2.3 (0.6)	3.1 (1.5)
3-OH-C _{16,0}	2.3 (0.7)	2.3 (0.6)	3.0 (0.9)	2.6 (0.8)
3-OH-C _{16,0}	23.8 (3.2)	22.6 (4.6)	24.5 (3.0)	26.2 (11.1)
3-OH-C _{17,0}	32.5 (7.5)	25.4 (9.8)*	43.5 (5.4)	42.8 (11.9)
3-OH-C _{17,0}	4.1 (1.8)	5.0 (2.8)	5.1 (2.8)	4.1 (2.1)

Healthy periodontia, smokers vs. non-smokers: * $p < 0.05$.

Chronic periodontitis, smokers vs. non-smokers: # approaching significance ($p = 0.051$).

* $p < 0.05$.

3-OH-C_{12,0}, 3-OH-C_{13,0}, and 3-OH-C_{14,0} percentages in smokers shifted by -50.0, +63.2, and -15.8%, respectively, relative to non-smokers.

and C17). The largest shifts were noted for 3-OH C14 (3-OH-tetradecanoic) and 3-OH C17 (3-OH-iso-heptadecanoic). 3-OH C14 comprised 23.4% (7.3%) of the total fatty acids in health but only 15.0% (4.0%) in individuals with CP. 3-OH C17 comprised 29.4% (9.1%) of the total fatty acids in health, which was elevated to 43.1% (9.7%) in the group with disease.

Salivary 3-OH Fatty Acid Profiles in Periodontally Healthy Smokers and Non-smokers

The 3-hydroxy fatty acid composition [relative percentage; mean (\pm SD)] of smokers and non-smokers with healthy periodontia is presented in Table 3. A significant relative decrease in 3-OH-C_{13,0} was accompanied by an increase in 3-OH-C_{17,0} in smokers compared with non-smokers.

Salivary 3-OH Fatty Acid Profiles in Smokers and Non-smokers with Chronic Periodontitis

Typical 3-OH fatty acid profiles of saliva from a current non-smoker with chronic periodontitis and a smoker with chronic periodontitis are shown in Figure 1.

The 3-hydroxy fatty acid composition [relative percentage; mean (\pm SD)] of smokers and non-smokers with CP is also presented in Table 3. The most prominent shifts in smokers, compared with non-smokers, occurred in the short, straight-chain lipid A fatty acids, 3-OH C12, C13, and C14. The high-potency, pro-inflammatory C12 and 14 3-OH fatty acids dropped from 2.7% (1.3%) and 16.5% (3.6%), respectively, in current non-smokers to 1.8% (1.1%) and 13.9% (3.9%), respectively, in individuals who smoked.

DISCUSSION

We hypothesized that smoking induces alterations to the structure of lipid A in a manner consistent with reduced inflammatory potential. We have previously shown that lipid A moieties in saliva are diagnostic for periodontitis with remarkable accuracy (Ferrando *et al.*, 2005). Indeed, the sensitivity, specificity, positive predictive value, and negative predictive value of 3-OH FA

analysis in the diagnosis of periodontitis were, respectively, 0.92, 1.00, 1.00, and 0.90, suggesting that salivary LPS levels reflect alterations to lipid A content of plaque. While mucosal bacteria will certainly contribute to the salivary LPS profile, signals associated with subgingival, anaerobic, Gram-negative periodontal pathogens are robust (Ferrando *et al.*, 2005). Several groups have shown that subgingival bacteria can be readily monitored in saliva and correlate with disease status (Martinez-Pabon *et al.*, 2008; Bagyi *et al.*, 2009; Van Assche *et al.*, 2009). Thus, we considered saliva an adequate substrate with which to begin to examine the influence of tobacco on oral lipid A structures.

Of particular interest were the branched (iso)-chain 3-OH fatty acids produced by many of the anaerobic Gram-negative bacteria associated with periodontitis, such as *Bacteroides*, *Tannerella*, *Porphyromonas*, and *Prevotella* spp. (Bronz and Olsen, 1991; Sakamoto *et al.*, 2002), as well as the fatty acid chains associated with maximal lipid A inflammatory potential, *i.e.*, C12 and C14 fatty acids (Berezow *et al.*, 2009).

Of the key periodontal pathogens (Holt and Ebersole, 2005), *P. gingivalis* and *T. forsythia* produce 3-OH-C_{17,0} as a major 3-OH FA (Bronz and Olsen, 1991; Sakamoto *et al.*, 2002), whereas, while it is still somewhat controversial, *T. denticola* may not produce hydroxy fatty acids (Dahle *et al.*, 1996; Schultz *et al.*, 1998; Wyss, 2007). Disease-associated *Capnocytophaga* spp. (Loesche and Grossman, 2001) have also been reported to produce predominantly 3-OH-C_{17,0} (Ferrando *et al.*, 2005). Such pathogen-associated lipid A signals predominate in the saliva of individuals with periodontitis, where 3-OH-C_{17,0} represents 43.1% (9.7%) of the total fatty acids. These data are consistent with our earlier work that has shown that 3-OH FA analysis shows great promise in the diagnosis of periodontitis (Ferrando *et al.*, 2005).

While we cannot be certain that the fatty acids in saliva are entirely derived from lipid A, 3-OH fatty acids are routinely used as specific LPS markers in environmental research. In blood, 3-OH fatty acids can stem either from LPS or from mammalian fatty acid metabolism, but concentrations of the latter are comparatively low (Szponar *et al.*, 2002). In addition, odd-chain 3-OH FAs such as 3-OH 13 and straight- and branched-chain 3-OH C17 acids are thought to be derived exclusively from LPS (Ferrando *et al.*, 2005).

The relative percentages of most fatty acids monitored were similar in smokers and non-smokers with periodontitis. However, the relative percentages of the high-potency, pro-inflammatory C12 and 14 3-OH fatty acids were lower in current smokers [1.8% (1.1%) and 13.9% (3.9%), respectively] compared to those in current non-smokers [2.7% (1.3%) and 16.5% (3.6%)]. This corresponds to a drop of 33% and 16%, respectively. These data are noteworthy for two primary reasons. First, C12 and 14 3-OH fatty acids are more commonly found in the LPS of early colonizers and commensals, such as *Neisseria*, *Haemophilus*, and *Veillonella* spp., than in overt periodontal pathogens (Ferrando *et al.*, 2005). Perhaps more importantly, C12 and 14 3-OH fatty acids represent the consensus chain length in the most potent pro-inflammatory structural lipid A isoform identified to date, *E. coli* LPS (Berezow *et al.*, 2009).

At this stage, it is not possible to determine if tobacco smoke directly alters the types of fatty acids metabolized into LPS during the biosynthetic process or simply influences the types of bacteria contributing to the overall fatty acid profile in saliva. It is known that members of pathogenic periodontal microflora can produce heterogeneous lipid A molecules with variable inflammatory capabilities (Dixon and Darveau, 2005; Al-Qutub *et al.*, 2006; Bainbridge *et al.*, 2006). The mechanisms contributing to altered fatty acid length are only beginning to be unraveled, e.g., *E. coli* expressing *P. gingivalis* acyltransferases (lpxA and lpxD) transfer longer than normal fatty acid chains (Bainbridge *et al.*, 2006).

To summarize, bacterial succession likely explains the clear differences in 3-OH fatty acid profiles in the individuals with disease and the healthy individuals, particularly the increases in the long-chain fatty acids associated with anaerobic bacterial periodontopathogens, particularly 3-OH-C_{17:0} (147%, relative to controls). Significant reductions in the 3-OH fatty acids associated with the consensus (high potency) enteric LPS structure (3-OH-C_{12:0} and 3-OH-C_{14:0}; 33% and 16% reduction, respectively) were apparent in smokers compared with non-smokers with CP. Thus, smoking induces specific structural alterations to the lipid-A-derived 3-OH fatty acid profile in saliva that are consistent with an oral microflora of reduced inflammatory potential. Future studies, with larger populations, should establish if our observed differences between smokers and non-smokers remain significant after adjustment for demographic and clinical measures. These findings provide much-needed mechanistic insight into the established clinical conundrum of increased infection with periodontal pathogens but reduced clinical inflammation in smokers.

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